IJIE Int. J. Indust. Entomol. 37(1) 15-20 (2018)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2018.37.1.15

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Enzymatic activity of Endoplasmic Reticulum Oxidoreductin 1 from *Bombyx mori*

Kwanho Park[1], Eun-Young Yun[2] and Tae-Won Goo[3,*]

[1]*Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Wanju-gun 565-851, Korea*
[2]*Graduate School of Integrated Bioindustry, Sejong University, Seoul 05006, Korea*
[3]*Department of Biochemistry, School of Medicine, Dongguk University, Gyeongju 780-714, Korea*

Abstract

Most proteins produced in the endoplasmic reticulum (ER) of eukaryotic cells fold via disulfide formation (oxidative folding). Oxidative folding is catalyzed by protein disulfide isomerase (PDI) and PDI-related ER protein thiol disulfide oxidoreductases (ER oxidoreductases). In yeast and mammals, ER oxidoreductin-1s (ERO1s) supply oxidizing equivalent to the active centers of PDI. We previously identified and characterized the ERO1 of *Bombyx mori* (bERO1) as a thioredoxin-like protein that shares primary sequence homology with other ERO1s. Here we compare the reactivation of inactivated rRNase and sRNase by bERO1, and show that bERO1 and bPDI cooperatively refold denatured RNase A. This is the first result suggesting that bERO1 plays an essential role in ER quality control through the combined activities of bERO1 and bPDI as a catalyst of protein folding in the ER and sustaining cellular redox homeostasis.

© 2018 The Korean Society of Sericultural Sciences
Int. J. Indust. Entomol. 37(1), 15-20 (2018)

Received : 20 Jun 2018
Revised : 27 Aug 2018
Accepted : 11 Sep 2018

Keywords:
Bombyx mori,
endoplasmic reticulum oxidoreductin 1 (ERO1),
protein disulfide isomerase (PDI),
disulfide bond,
baculovirus expression vector system (BEVS)

Introduction

The endoplasmic reticulum (ER) is a highly specialized organelle involved in the maturation of extracellular membrane proteins and secreted proteins. Disulfide bond formation is a key step in this process (Sevier and Kaiser, 2002). Disulfide bonds are usually formed by pairing and oxidative linkage of sulfhydryl groups (-SH) on cysteine residues during the folding process in the ER. Cooperative activity of two proteins, protein disulfide isomerase (PDI) and flavin adenine dinucleotide (FAD)-dependent oxidase ER oxidoreductin 1 (ERO1) formed disulfide bonds, which both have protein oxidation with redox reactions (Schroder, 2008; Sevier and Kaiser, 2006). Two proteins use an exchanging mechanism of thiol-disulfide to transfer disulfide bonds on to their substrate proteins (Sevier and Kaiser, 2006). Disulfide bonds formed between cysteines in the active site of PDI are transferred directly to the folding secretory protein. ERO1 reoxidizes reduced PDI, whereas reduced ERO1 is reoxidized by its FAD cofactor (Jessop *et al*., 2004; Tu and Weissman, 2004; Frand and Kaiser, 1999). The ERO1 contains a conserved C-X-X-X-X-C motif (N-terminal) and C-X-X-C-X-X-C motif (C-terminal). The N-terminal C-X-X-X-X-C motif likely transfers electrons to the two latter residues of the C-X-X-C-X-X-C motif (C-terminal), which are in close proximity to the isoalloxazine ring of FAD (Dias-Gunasekara *et al*., 2005).

We previously isolated ERO1 homolog from Bombyx mori

***Corresponding author.**
Tae-Won Goo
Department of Biochemistry, School of Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea
Tel: +82-31-290-8532 / FAX: +82-54-703-7801
E-mail: gootw@dongguk.ac.kr

© 2018 The Korean Society of Sericultural Sciences

(bERO1I), which was highest in posterior silk gland on the sixth day of the 5th instar larvae. The cDNA contained an open reading frame of 489 amino acids and a predicted size of 57.4 kDa. that encompasses two conserved redox active motifs, a C-X-X-X-X-C motif of N-terminal and C-X-X-C-X-X-C motif of C-terminal. Drugs that increase ER stress (e.g., antimycin, calcium ionophore A23187, dithiothreitol, H_2O_2, monencin, and tunicamycin) increased bERO1 mRNA expression. In addition, expression levels of bERO1 exactly coincided with that of bPDI. In our previous study, we did not measure the bERO1 activity (Seo *et al.,* 2015). Therefore, in this study, we generated bERO1 recombinant proteins and tested their ERO1 activity using an RNase activity assay.

Materials and Methods

Experimental cells

Sf9 cells, derived from *Spodoptera frugiperda*, were cultured at 27℃ in TC-100 medium containing 10% (v/v) heat-inactivated fetal bovine serum, as described previously (Goo *et al*., 2008).

Construction of Expression vector for bERO1 and Western blot analysis

The cDNA encoding bERO1 was amplified using a sense primer (5'-GAATTCCTGTCATGGCGCGGACATCCA-3'; underline indicates the initial codon) and an antisense primer (5'-CTCGAGCCGCCTAAGCATGATTCTGAAATT-3'; underline indicates the stop codon that was modified from the original TAA). The PCR products were ligated once to a TA cloning vector, pGEM-T (Promega). The pGEM-bERO1 was digested with *Eco*R I / *Xho* I and subcloned into the baculovirus vector, pBAC-gus-1 (Novagen) to form pBACgus1-bERO1. After incubation for 15 min, a mixture pBAC-gus-1-bERO1/ BacVector-3000 Triple Cut Virus DNA (Novagen, Madison, WI) with Eufectin (Novagen) was inoculated at 27℃ for 5 h at the Sf-9 cell line. The infected cells were collected after 3 days and digested with lysis buffer (6.25 mM Tris (hydroxymethyl) aminomethane-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% DTT). Electrophoresis was performed in 7.5% polyacrylamide gels. Protein transfer to a PVDF membrane was carried out in a semi-dry system from Bio-Rad.. Immunological detection was done by first incubating the PVDF membrane for 1 h at room temperature with the primary antiserum in TBS containing 0.05% Tween 20. Subsequently, the PVDF membrane was incubated for 1 h with peroxidase-conjugated secondary immunoglobulins. The PVDF membrane was washed intensively between each step in 150 mM NaCl, 10 mM Tris (hydroxymethyl) aminomethane-HCl, pH 7.5, 2% non-fat dry milk.

Purification of the bERO1-His_6 fusion protein

Recombinant bERO1 proteins were purified from Sf9 cells infected with recombinant baculoviruses by IMAC using a Ni^{2+}-immobilized resin (His-Trap chelating column; Amersham Pharmacia Biotech., Uppsala, Sweden). The column was equilibrated with phosphate start buffer (20 mm phosphate, 0.5 mM NaCl, and 10 mM imidazole, pH 7.4), and the homogenized/clarified sample was applied. The column was then washed with phosphate buffer and sample fractions (0.5 mL each) were collected. The recombinant proteins were eluted with a phosphate buffer containing increasing amounts of imidazole (50, 100, 200, 300, 400, and 500 mM). The fractions were monitored at A_{280} using the corresponding elution buffer as a blank, and detected based on their fluorescence intensity using a fluorescence spectrometer. Most of the bound proteins were eluted between 100 and 200 mM imidazole.

RNase activity assay

PDI activity was measured by the ability to renature reduced or scrambled RNase A (rRNase and sRNase). The reactivation of denatured RNase A was monitored as described by Lyles and Gilbert (1991) with modifications. In brief, 5 mg of RNase A (Type XII-A, Sigma-Aldrich) were reduced overnight at 25 ℃ in 1 mL of 100 mM Trisacetate (pH 8.0), 4 mM EDTA, 6 m guanidine hydrochloride, and 140 mM DTT. Then, guanidine hydrochloride and DTT were removed by passage through a Bio-Gel P4 column (Bio-Rad, Hercules, USA) that had been equilibrated with 0.1% acetic acid. The scrambled RNase A, which had exchanged cysteines, was purchased from TaKaRa (Shiga, Japan). Activation of the reduced and scrambled RNase was assayed by mixing it with each purified bPDI and ERO1, and measuring the rate of refolded RNase in small aliquots. Briefly, 0.5 mg/mL reduced and scrambled RNase were mixed with 5 μg/mL bPDI or 5 μg/mL bERO1 and 10 μM DTT in 50

mM sodium phosphate buffer (pH 7.5) and incubated at 30℃. RNase was subsequently added at a concentration of 50 μg/mL. Aliquots were removed at several time points and the absorbance was measured at 260 nm.

Results and Discussion

A *Bombyx mori* ERO1 homologue (bERO1) was isolated from the culture cell line Bm5, which was treated with tunicamycin using a differential screening method, and both strands sequenced the cDNA clone (Hoog, 1991). The sequence data of the bERO1 was submitted to Genbank under the accession number FJ502246. Although a cDNA encoding bERO1 shows a high sequence variation compared with known ERO1 cDNAs, the bERO1 protein has two conserved redox active motifs, a C-X-X-X-X-C motif of N-terminal and C-X-X-C-X-X-C motif of C-terminal, which is similar to the other ERO1s.

To investigate whether bERO1 is a functional homologue of ERO1, it is necessary that the bERO1 cDNA be correctly translated to a protein, because many cDNAs are expressed alternatively or are pseudogenes that do not encode proteins. We cloned a PCR product encoding the full ORF of bERO1 into a pGEM-T vector. After a EcoR I and Xho I digestion, the bERO1 fragment was subcloned into the baculovirus transfer vector pBAC-gus-1 to form pBAC-gus-1-bERO1 (Fig. 1).

To confirm whether or not the cDNA encoding bERO1 translates correctly *in vivo* by bERO1, the recombinant baculovirus (vAc-bERO1) was translated in the culture insect Sf-9 cell lines. As shown in Fig. 2, the cDNA encoding the *Bombyx mori* ERO1 homologue was successfully translated in bERO1 (line 3 in panel a), which was also recognized by anti-mouse ERO1 antibodies (line 3 in panel b). No band estimated PDI appeared between the wild type cells and the cells infected with the wild type baculovirus (line 1, 2 in panel b). The result suggests that although the bERO1-cDNA isolated in this study shows a low DNA sequence homology among the known bERO1s, the bERO1 protein shares the same antigen domains

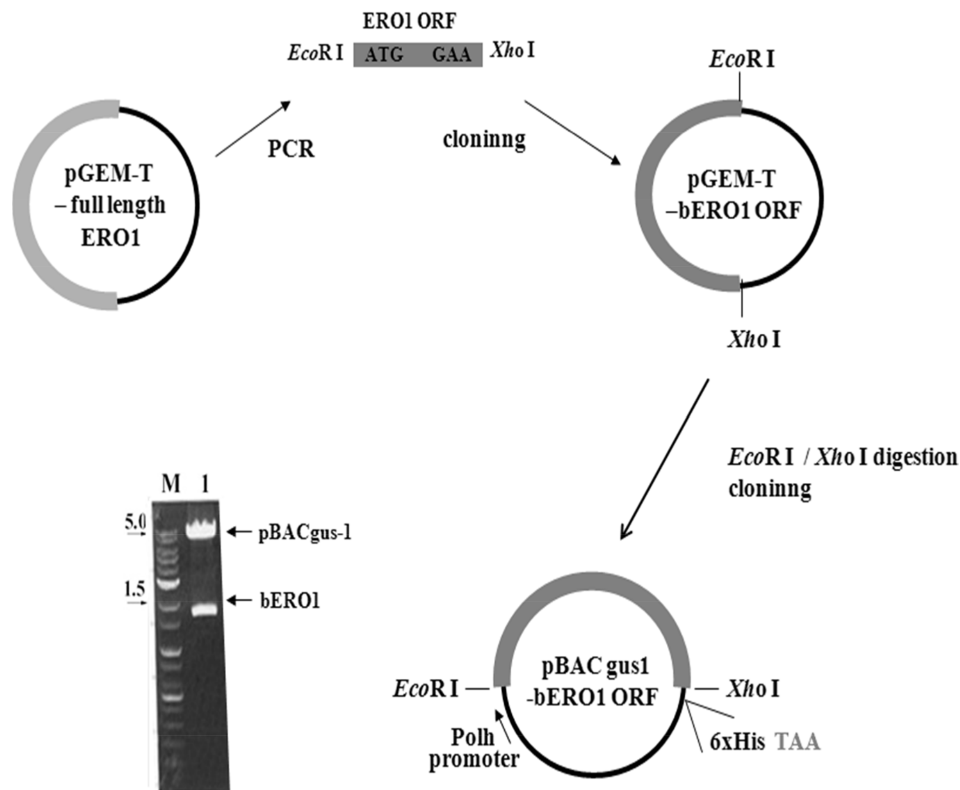

Fig. 1. Constuction of baculovirus transfers vector pBACgus1-bERO1 ORF. The PCR product for ORF flanked by *Eco*R Ⅰ and *Xho* Ⅰ site were inserted into pGEMT vector. The pGEMT-bERO1 ORF was digested with *Eco*R Ⅰ and *Xho* Ⅰ, and then the DNA frgment was cloned into pBACgus1 vector. The pBACgus1-bERO1 ORF was digested with *Eco*R Ⅰ and Xho Ⅰ (lane 1). M, 1 kb ladder marker.

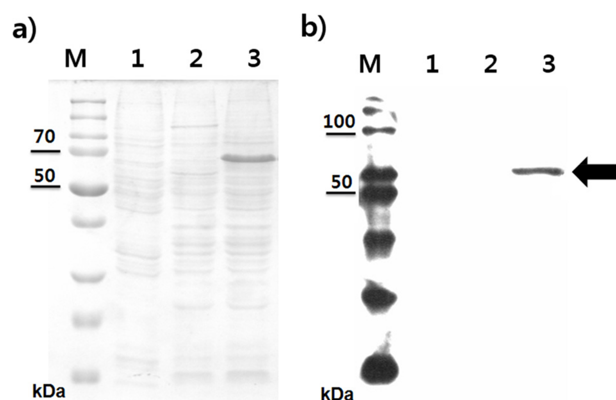

Fig. 2. (a) SDS-PAGE and (b) Western blot analysis of bERO1 protein. Sf-9 cells (3.0×10^6) were infected with recombinant virus encoding bERO1/His_6 at a total m.o.i. of 5. cells were harvested 96 hr after infection (lane 3). Western blot analysis was performed, using a mouse anti-ERO1 antibody. Lane 1, protein extracted from Sf-9 normal cells ; lane 2, protein extracted from cells infected with wild type virus ; lane M, Protein size marker. Arrow indicates bERO1 protein. bERO1 protein size is about 57 kDa.

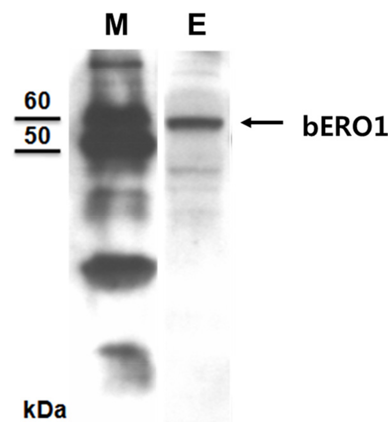

Fig. 3. Purification and Western blot analysis of bERO1. Sf9 cells were infected with a recombinant baculovirus (vAc-bERO1) encoding bERO1-His_6 at a total m.o.i. of 5. The cells were harvested 72 h after infection and the recombinant protein was purified. Western blot analysis was performed using rat anti-ERO1 antibodies, with bERO1 indicated by an arrow. M, protein size marker; E, bERO1 protein.

with another ERO1 that is recognized by anti-mouse ERO1 antibodies.

In the case of eukaryote, oxidative protein folding in the ER is an essential function that requires the electron relay system between the proteinaceous components of the pathway (Zito, 2015). During this process, PDIs directly oxidize new substrate proteins and are subsequently reduced. ERO1 is located upstream of this redox reaction, which reoxidizes and reactivates one of two thioredoxin-like domains of PDI to induce a new cycle of oxidative protein folding through its cofactor FAD (Pollard *et al*., 1998; Frand and Kaiser, 1998; Tu and Weissman, 2002). ERO1 activity plays an essential role in disulfide bond formation in simple eukaryotes such as yeast and worms. ERO1 function is essential for disulfide bond formation in these types of simple eukaryotes but is largely compensated for by alternative pathways in mammals (Zito *et al*., 2010a; Zito *et al*., 2010b). Although ERO1 participates in alternative mammalian pathways, its importance is highlighted by UPR-mediated up-regulation (Zito, 2015; Harding et al., 2003). Formation of disulfide bonds within the ER requires the combined activities of ERO1 and PDI (Baker *et al*., 2008).

In this study, we investigated whether or not bPDI and bERO1 are required for oxidative protein folding in the ER as well as mammals. To investigate the relationship between bERO1 and bERO1 enzymatic activity in *B. mori*, we first expressed His6-tagged bERO1 (bERO1-His_6) via recombinant baculovirus (vAc-bERO1) transfection into Sf9 cells. The recombinant bERO-His_6 proteins were purified by IMAC using a Ni^{2+}-immobilized resin and detected with anti-mouse ERO1 antibodies which have already demonstrated their specificity for bERO1 (Fig. 3). Recombinant bPDI proteins were obtained from previous studies (Goo *et al*., 2008).

We next used the reduced and scrambled RNase folding method to test the enzymatic activity of bERO1 and bPDI (Xiao *et al*., 2001). The ability to refold reduced and denatured RNase molecules is a classic measure of ERO1and PDI. RNase A showed time-dependent, spontaneous refolding and recovery of function after reduction (Fig. 2, left panel) and scrambling (Fig. 2, right panel). Recombinant bERO1 and bPDI combined treatment restored the RNase activity to 89% (reduced) and 79% (scrambled) of the control level, compared to 72% and 66%, respectively, for bPDI alone treatment (Fig. 4).

Thus, the ERO1 family member bERO1 may play an important role in protein folding and assembly in the ER of insect cells. Understanding the enzymatic activity of bEROI and bPDI will facilitate the production of heterologous proteins in eukaryotic environments, since heterologous proteins frequently form insoluble aggregates or are improperly folded in the ER. In addition, a better understanding of the in vivo protein folding will benefit the

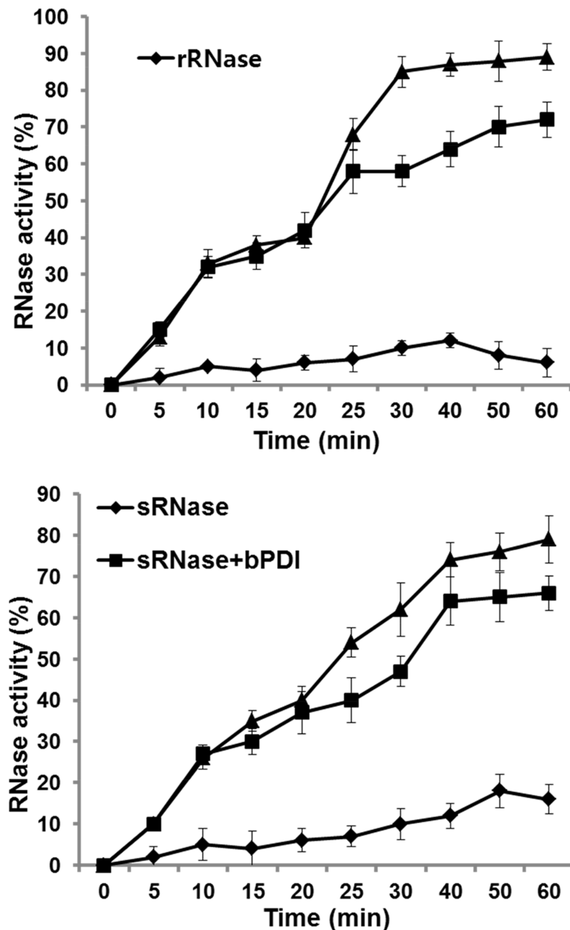

Fig. 4. Enzymatic activity of the recombinant bERO1 protein. The assay mixture contained 10 μM DTT, 0.5 mg/mL reduced (rRNase) and scrambled (sRNase) RNase with 5 μg/mL purified recombinant bERO1, purified recombinant bERO1 and bPDI, or nothing else in 50 mM phosphate buffer (pH 7.5). RNase activity was measured at the indicated time points. The graph shows the percentage of refolding mediated by recombinant bERO1, or recombinant bERO1 and bPDI. All experiments were performed 3 times and the graphs indicate the averages.

field of protein engineering and help to unravel the molecular basis of diseases associated with protein misfolding.

Acknowlegements

This study was supported by the Bio-industry Technology Development Program (318018-3) through the Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, and by the Cooperative Research Program for Agriculture Science & Technology Development (PJ01084302) through the Rural Development Administration, Republic of Korea.

References

Baker KM, Chakravarthi S, Langton KP, Sheppard AM, Lu H, Bulleid NJ (2008) Low reduction potential of Ero1alpha regulatory disulphides ensures tight control of substrate oxidation. EMBO J 27, 2988-2997.

Dias-Gunasekara S, Gubbens J, van Lith M, Dunne C, Williams JA, Kataky R, *et al.* (2005) Tissue-specific expression and dimerization of the endoplasmic reticulum oxidoreductase Ero1beta. J Biol Chem 280, 33066-33075.

Frand AR, Kaiser CA (1998) The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol Cell 1, 161-170.

Frand AR, Kaiser CA (1999) ERO1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol Cell 4, 469-477.

Goo TW, Yun EY, Kim SW, Choi KH, Kang SW, Shin KS, *et al.* (2008) Domain a' of Bombyx mori protein disulfide isomerase has chaperone activity. Z Naturforsch C 63(5-6):435-9.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, *et al.* (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 11, 619-633.

Hoog C (1991) Isolation of a large number of novel mammalian genes by a differential cDNA libraryn screening strategy. Nucleic Acids Res 19, 6123-6127.

Jessop CE, Chakravarthi S, Watkins RH, Bulleid NJ (2004) Oxidative protein folding in the mammalianendoplasmic reticulum. Biochem Soc Trans 32, 655-658.

Lyles MM, Gilbert HF (1991) Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: pre-steady-state kinetics and the utilization of the oxidizing equivalents of the isomerase. Biochemistry 30, 619-625.

Pollard MG, Travers KJ, Weissman JS (1998) ERO1p: A novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol Cell 171-182.

Schroder M (2008) Engineering eukaryotic protein factories. Biotechnol Lett 30, 187-196.

Seo M, Ryou HJ, Yun EY, Goo TW (2015) Molecular Characterization of Endoplasmic Reticulum Oxidoreductin 1 from Bombyx mori. Int J Mol Sci,16(11), 26520-26529.

Sevier CS, Kaiser CA (2002) Formation and transfer of disulphide bonds in living cells. Nat Rev Mol Cell Biol 3, 836-847.

Sevier CS, Kaiser CA (2006) Disulfide transfer between two conserved cysteine pairs imparts selectivity to protein oxidation by Ero1. Mol

Biol Cell 17, 2256-2266.

Tu BP, Weissman JS (2002) The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. Mol Cell 10, 983-994.

Tu BP, Weissman JS (2004) Oxidative protein folding in eukaryotes: Mechanisms and consequences. J Cell Biol, 164, 341-346.

Xiao R, Solovyov A, Gilbert HF, Holmgren A, Lundström-Ljung J (2001) Combinations of proteindisulfide isomerase domains show that there is little correlation between isomerase activity and wild-type growth. J Biol Chem 276, 27975-27980.

Zito E (2015) ERO1: A protein disulfide oxidase and H2O2 producer. Free Radic Biol Med 83, 299-304.

Zito E, Chin KT, Blais J, Harding HP, Ron D (2010a) ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. J Cell Biol 188, 821-832.

Zito E, Melo EP, Yang Y, Wahlander A, Neubert TA, Ron D (2010b) Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. Mol Cell 40, 787-797